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# Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A

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## Summary

Sperm motility is regulated by a complex balance between kinases and phosphatases. Among them, phosphatidylinositol 3-kinase (PI 3-kinase) has been recently suggested to negatively regulate sperm motility (Luconi, M., Marra, F., Gandini, L., Lenzi, A., Filimberti, E., Forti, G. and Baldi, E. (2001). *Hum. Reprod.* 16, 1931-1937). We demonstrate the presence and activity of PI 3-kinase in human spermatozoa and have investigated the molecular mechanism(s) by which the PI 3-kinase inhibitor, LY294002, triggers an increase in sperm motility. PI 3-kinase inhibition results in an increase in intracellular cAMP levels and in tyrosine phosphorylation of the protein kinase A-anchoring protein AKAP3. These effects finally result in a stimulation of protein kinase A (PKA) binding to AKAP3 in sperm tails through the regulatory subunit RII $\beta$ . The increased binding of RII $\beta$  to AKAP3 induced by LY294002 is mainly due to tyrosine phosphorylation of

AKAP3, since it is completely blocked by the tyrosine kinase inhibitor erbstatin, which also reverses the effects of LY294002 on motility and suppresses PKA-AKAP3 interaction. The requirement of PKA binding to AKAP3 for sperm motility is confirmed by the reduction of motility induced by an inhibitor of RII $\beta$ -AKAP3 binding, Ht31, whose effects on sperm motility and PKA binding to AKAP3 are reversed by LY294002.

These results demonstrate that PI 3-kinase negatively regulates sperm motility by interfering with AKAP3-PKA binding, providing the first evidence of a molecular mechanism by which PKA can be targeted to sperm tails by interaction with tyrosine phosphorylated form of AKAP3.

Key words: Sperm, Motility, AKAP, PKA, Phosphatidylinositol 3-kinase, Phosphorylation

## Introduction

Motility is one of the most peculiar functions of the mature male gamete. In mammalian spermatozoa, the ability to actively swim, based on the specialized structure of the flagellum, is acquired during the transit through the epididymis under the control of different factors, such as cAMP, intracellular pH, intracellular calcium and phosphorylation of sperm proteins (Leclerc et al., 1996; Ashizawa et al., 1995; Vijayaraghavan et al., 1985; Vijayaraghavan et al., 1997a). Such factors are also involved in the development of a specific type of motility, called hyperactivation, which occurs as a consequence of sperm activation in the female genital tract or by in vitro incubation in various media (Ho and Suarez, 2001). Sperm protein phosphorylation is regulated by a complex balance between kinases and phosphatases, which play a pivotal role in the development and maintenance of motility (Vijayaraghavan et al., 1997a; Tash and Bracho, 1994). In particular, the cAMP/PKA-dependent pathway has been demonstrated to be involved in tyrosine phosphorylation of different sperm proteins in the flagellum associated to an increase in sperm motility (Horowitz et al., 1988; Leclerc et al., 1996; Vijayaraghavan et al., 1997a; Carrera et al., 1996; Si and Okuno, 1999; Patil et al., 2002; Leclerc and Goupil, 2002; Ficarro et al., 2003). Both processes are stimulated by cAMP

analogues and through inhibition of serine-threonine phosphatases by calyculin A (Ashizawa et al., 1995). cAMP produced by activation of adenylate cyclase binds to PKA holoenzyme resulting in activation of the catalytic (C) subunit after dissociation from the regulatory one (R). Multiple regulatory subunits (RI  $\alpha$  and  $\beta$ , RII  $\alpha$  and  $\beta$ ) have been characterized in spermatozoa, although those associated with the insoluble structures of the sperm flagellum are predominantly of type II (Lieberman et al., 1988; Horowitz et al., 1988; Vijayaraghavan et al., 1999), and seem to be implicated in regulation of sperm motility, at least in humans (Vijayaraghavan et al., 1997b; Burton et al., 1999). These regulatory subunits not only modulate PKA serine-threonine kinase activity, but can also target the catalytic subunit to different cell compartments by interacting with protein kinase A-anchoring proteins, AKAPs. Therefore, while cAMP levels temporally regulate PKA, spatial regulation within the cell occurs through compartmentalization by binding to AKAP, thus assuring specificity of PKA function. Since AKAPs can also simultaneously bind other transduction molecules including many PKA substrates, they also act as scaffolding proteins coordinating interactions between different signaling pathways (Moss and Gerton, 2001). AKAPs possess a variety of binding affinities for the different subtypes of R isoforms,

although the molecular mechanisms underlying modulation of these affinities have still to be elucidated. Yet, the RII binding domain of different AKAPs is highly conserved among species (Carrera et al., 1996; Vijayaraghavan et al., 1997b; Moss et al., 1999; Jha and Shivaji, 2002).

Among the enzymes involved in regulation of sperm motility, phosphatidylinositol 3-kinase (PI 3-kinase) has been recently suggested to play an important role, since its pharmacological inhibition by LY294002 results in a significant increase in human sperm forward motility (Luconi et al., 2001). This family of dimeric enzymes, consisting of a catalytic (110 kDa) and a regulatory subunit (85 kDa), is widely expressed in somatic cells and phosphorylates the inositol ring of different phosphoinositides in position three, and also stimulates serine-threonine kinases (Wymann and Pirola, 1998). We have investigated the signaling mechanisms involved in LY294002 stimulation of human sperm motility focusing in particular on the AKAP-PKA system. We found that inhibition of PI 3-kinase results in an increase in intracellular cAMP levels, in tyrosine phosphorylation of AKAP and PKA recruitment to sperm tails, providing evidence for a more general mechanism through which tyrosine phosphorylation of AKAP may influence PKA recruitment to specific cell compartments.

## Materials and Methods

### Antibodies and chemicals

All reagents for human sperm preparation were from Irvine (Santa Ana, CA, USA). All reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy). The conjugated secondary antibodies, protein A- and G-Sepharose and other not specified reagents were from Sigma Chemical Co (St Louis, Missouri, USA). Peroxidase-conjugated and unconjugated PY20 antibodies, LY294002, PKA inhibitor H89 and dibutyl cAMP (dbcAMP) were obtained from Calbiochem (La Jolla, CA, USA). [ $\gamma$ - $^{32}$ P]ATP and the RIA kit for cAMP measurement were from NEN Life Science (Boston, MA, USA). Anti-PI 3-kinase, anti-RII antibodies and serine/threonine phosphatase activity kit were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-AKAP3, Ht31 and P-Ht31 were kindly provided by Prof. Daniel Carr (Veteran Affairs Medical Center, Oregon Health Science University, Portland, Oregon, USA). Purified PKA regulatory subunit RII  $\alpha$  and  $\beta$  were from Promega (Madison, WI, USA). Anti-ERK2 antibody was kindly provided by Prof. M. Dunn (Medical College of Wisconsin, Milwaukee, WI, USA). Anti-FSP95 antibody was kindly provided by Prof. John Herr (Charlottesville, VA, USA). LY303511 was kindly provided by Ely Lilly (Indianapolis, IN, USA).

### Preparation of spermatozoa

Human semen was collected in our laboratory according to the WHO recommended procedure (WHO, 1999) by masturbation from men undergoing semen analysis for couple infertility caused by a female factor. All the experiments were performed using samples from these men who met all the WHO criteria for normozoospermia (WHO, 1999). Samples with leukocytes and/or immature germ cell concentration greater than  $10^6$ /ml were not included in the study. Semen samples were processed by swim up or by minipercoll techniques as previously described (Krausz et al., 1996). Briefly, for swim up selection, 1 ml aliquots of semen were gently layered with 1 ml of 1% human serum albumin-HTF (HSA-HTF) medium and incubated at 37°C, 5% CO<sub>2</sub>. After 1 hour, 800  $\mu$ l of the upper medium

phase were collected and checked for sperm count and motility. For the minipercoll technique, semen samples were layered on top of a 95, 75 and 50% percoll gradient (Pure sperm, Irvine, Santa Ana, CA, USA) and centrifuged at 500 g for 30 minutes. Only spermatozoa migrating in the lower phase were collected. After washing, spermatozoa were incubated for the indicated times with the stimuli. In some experiments ejaculated spermatozoa were treated with different stimuli and motility was evaluated according to WHO manual (WHO, 1999).

Preparation of sperm tail and head purified fractions was performed according to the method of Reinton et al. (Reinton et al., 2000) with slight modifications. Briefly, after washing, treated spermatozoa were sonicated at 8 burst 3 $\times$ 10 seconds in PBS on ice. After 2000 rpm centrifugation for 5 minutes, supernatant, corresponding to tail fractions, and pellet, corresponding to head fractions, were checked under microscope and then extracted in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethyl-sulfonyl fluoride (PMSF)).

### Indirect immunofluorescence microscopy of human spermatozoa

Swim-up-selected human spermatozoa were layered on slides, fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton X-100 and subsequently incubated for 2 hours in a humidity chamber at 4°C with blocking solution (2.5% BSA, 5% goat serum in TTBS; Tris-buffered saline containing 0.1% Tween 20, pH 7.4) followed by primary anti-p85 PI 3-kinase antibody revealed by TRITC-conjugated anti-rabbit secondary antibody (1 hour incubation, room temperature, dilution 1:50 in TTBS). Slides were rinsed with TBS and mounted in a glycerol:PBS solution (1:9, pH 8.5). Negative controls were performed by substituting blocking buffer for the primary antibody. The cells were observed under a Leica immunofluorescence microscope.

### SDS-PAGE and western blot analysis

After the different treatments, sperm samples were processed for SDS-PAGE as previously described (Luconi et al., 1995). Briefly, sperm samples, containing about 5 $\times$ 10<sup>6</sup> cells/ml were washed and resuspended in lysis buffer. After protein measurement (Coomassie kit, Bio-Rad Laboratories, Hercules, CA, USA), the sperm extracts, containing approximately 30  $\mu$ g of protein, were diluted in an equal volume of 2 $\times$  Laemmli's reducing sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2.5% pyronin and 200 mM dithiothreitol), incubated at 95°C for 5 minutes and loaded onto 8% or 10% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose (Sigma Co., St. Louis, MO, USA). In some experiments, equivalent protein loading was verified by staining parallel gels with Coomassie R. After blocking in either 5% BSA or skimmed milk for 2 hours in TTBS solution, nitrocellulose membranes were washed and then immunostained with primary antibodies followed by the relative peroxidase-conjugated secondary antibodies. The antibody-reacted proteins were revealed by the enhanced-chemiluminescence system (BM, Roche, Milan). For re-probing with different antibodies, the nitrocellulose membranes were washed for 30 minutes at 50°C in stripping buffer (10 mM Tris, pH 6.8, 2% SDS, 100 mM  $\beta$ -mercaptoethanol) and re-probed with specific primary antibodies.

### Immunoprecipitation analysis

To evaluate the presence of PI 3-kinase regulatory and catalytic subunits, about 50 million human spermatozoa, selected by the swim up technique, were resuspended in lysis buffer followed by immunoprecipitation. Briefly, after protein measurement, aliquots of cell lysates containing equal amount of proteins (300  $\mu$ g) were

incubated for 1 hour with 30  $\mu$ l of Protein A-Sepharose (or Protein G-Sepharose) for preclearing. Precleared lysates were then incubated for 1 hour using 3  $\mu$ g of the appropriate primary antibodies (anti-p85 PI 3-kinase regulatory subunit and anti-p110 PI 3-kinase catalytic subunit, respectively) followed by overnight incubation at 4°C with 50  $\mu$ l of Protein A-Sepharose. The immunobeads were washed three times in lysis buffer and then resuspended in 10  $\mu$ l of 2 $\times$  reducing sample buffer and subjected to SDS-PAGE followed by western blot analysis using anti-p85 PI 3-kinase or anti-p110 PI 3-kinase antibodies revealed by chemiluminescence. Lysates (300  $\mu$ g) of a prostate cancer (PC3) and a mammary cancer (MCF7) cell line were used as positive controls and immunoprecipitated by the same protocol.

Immunoprecipitation of AKAP3 was conducted as described above, using an anti-AKAP antibody. Since AKAP3 is detergent resistant because of its strong association with the fibrous sheath compartment, we used an SDS extraction method to lyse spermatozoa before performing immunoprecipitation (Dr P. Visconti, personal communication). Briefly, 50 million sperm were extracted in SDS-SDS-lysis buffer (20 mM Tris-HCl, pH 7.4, 0.2% SDS, 1 mM PMSF) for 30 minutes. After boiling for 5 minutes at 95°C, samples were kept for 10 minutes on ice and centrifuged at 12,000 rpm for 5 minutes at room temperature. Extracted supernatants were then subjected to AKAP3 immunoprecipitation (see above).

To evaluate the ability of Ht31 and LY294002 to modulate RII $\beta$  binding to AKAP3, RII $\beta$  co-immunoprecipitating with AKAP3 (same method as for immunoprecipitation, see above) was determined by western blot analysis using an antibody against RII $\beta$ .

#### RII overlay assay

Protein extracts (50  $\mu$ g) were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes as described above. RII binding to AKAP3 present in those extracts was investigated as previously described, by a modified western blot procedure, called overlay assay (Vijayaraghavan et al., 1997b; Rosenmund et al., 1994). Nitrocellulose membranes were incubated overnight in a blotting solution (Tris-buffered saline, pH 7.4, 5% milk, 0.1% BSA) containing purified RII $\alpha$  or RII $\beta$  (200 ng/ml). After washing, bound RII was detected by western blot analysis using antibodies against respectively RII  $\alpha$  or  $\beta$  followed by peroxidase-conjugated secondary antibodies revealed by chemiluminescence.

#### PI 3-kinase assay

Sperm samples treated as indicated, were extracted with lysis buffer A (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1% NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF). After measurement of proteins, aliquots of sperm extracts containing equivalent amount of proteins (300  $\mu$ g) were incubated for 1 hour with 50  $\mu$ l of Protein G-Sepharose for preclearing. Precleared lysates were then incubated for 1 hour using 3  $\mu$ g of rabbit anti-p85 PI 3-kinase (# 06-195, Upstate Biotechnology, Lake Placid, NY) on ice followed by overnight incubation at 4°C with 50  $\mu$ l of Protein A-Sepharose. Sepharose beads were washed twice in lysis buffer and twice with a 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA and 5 mM LiCl. At the last wash, all beads were divided into two groups: one quarter (1:4) was subjected to western blot analysis with the same antibody used for immunoprecipitation, to ensure that protein G pulls down the same amount of the enzyme in all samples. The remaining three-quarters (3:4) were suspended in kinase buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) containing 20  $\mu$ g of L- $\alpha$ -phosphatidylinositol (Sigma Chemical Co, St. Louis, Missouri, US) 25 mM MgCl<sub>2</sub> and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 60  $\mu$ l of 6 M HCl and 160  $\mu$ l of a mixture of chloroform and methanol (1:1) were added. Lipids were then resolved by thin layer chromatography plates (TLC

silica gel 60) (Merck Laborchimica, Florence, Italy) in chloroform, methanol, water and ammonium hydroxide (60:47:11,3:2). Dried TLC sheets were developed by autoradiography. Quantifications of the bands were performed using a Kodak image analysis system.

#### Intracellular cAMP levels

Sperm samples (4 $\times$ 10<sup>6</sup> cells) treated with 10  $\mu$ M LY294002 for 1 up to 15 minutes, were washed twice in PBS and extracted overnight in absolute ethanol at -20°C. Intracellular cyclic AMP levels were evaluated by a RIA kit (NEN Life Science, Boston, MA) in supernatants after centrifugation (1000 g, 10 minutes), lyophilization and reconstitution in 0.05 M sodium acetate buffer, pH 6.2 (Belen Herrero et al., 2000). Experiments were carried out in duplicate and results expressed as percentage of stimulation over control, taken as 100%.

#### Evaluation of sperm motility and viability

Motility was evaluated by computer assisted semen analysis (CASA, Hamilton Thorn Research, Beverly, MA, US). The settings used during CASA procedure were: analysis duration of 1 second (30 frames); minimum contrast, 80; minimum size, 3; low size and high size gates, 0.7 and 2.6; low intensity and high intensity gates, 0.34 and 1.40 (Luconi et al., 2001). All measurements were performed at 37°C. Motility was expressed as forward (type a+b motility, according to WHO manual, 1999) and rapid (type a motility, according to WHO manual, 1999).

Sperm viability was evaluated under a phase contrast light using the Eosin technique or the hyposmotic swelling test according to WHO manual (WHO, 1999).

#### Statistical analysis

Data are expressed as means  $\pm$  s.e.m.. Statistical analysis was performed with ANOVA and Student's *t*-test for unpaired and, when applicable, for paired data.

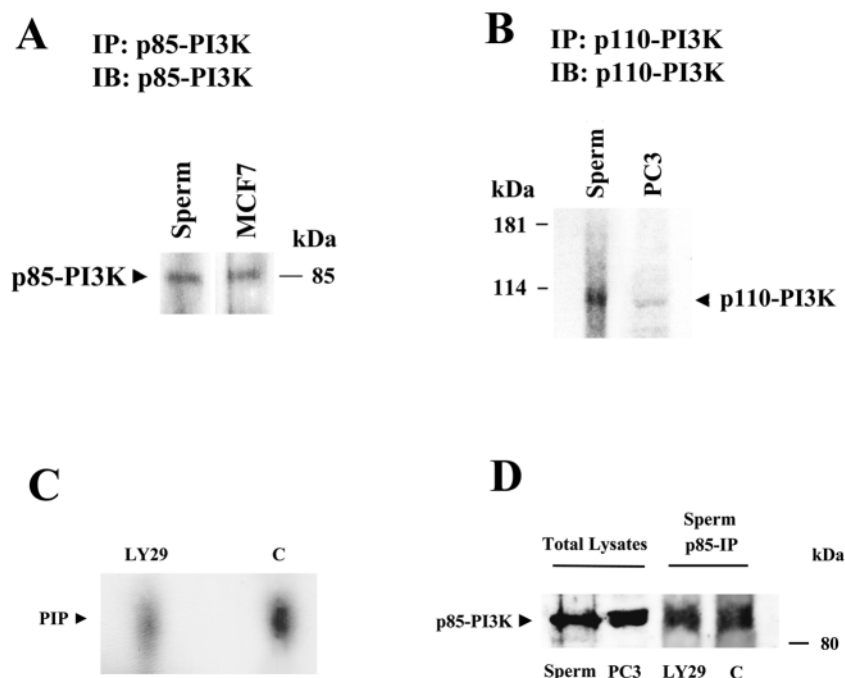
## Results

### Presence and activity of phosphatidylinositol 3-kinase (PI 3-kinase)

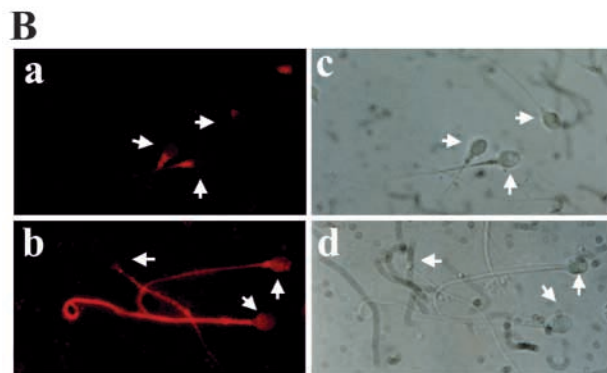
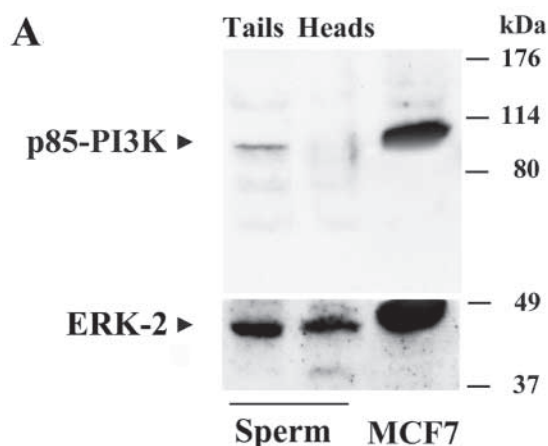
Although the presence of PI 3-kinase in human spermatozoa has been indirectly demonstrated by the use of inhibitors such as wortmannin and LY294002 (Luconi et al., 2001; Fisher et al., 1998), there has been no direct evidence of its presence and activity, so far. Thus, we performed immunoprecipitation of both regulatory and catalytic subunits from total lysates of capacitated human spermatozoa. As shown in Fig. 1A,B, both PI 3-kinase subunits are present in human spermatozoa migrating in SDS-PAGE at the expected molecular mass as in the positive controls (MCF7 cells, for p85 regulatory subunit and PC3 cells, for p110 catalytic subunit, respectively). PI 3-kinase specific activity was evaluated as the ability of the catalytic subunit, co-immunoprecipitated using a polyclonal antibody against p85, to phosphorylate phosphatidylinositol (PtdIns) in an *in vitro* kinase assay. As shown in Fig. 1C, PI 3-kinase activity in human sperm is inhibited by the treatment of sperm with 10  $\mu$ M LY294002. An aliquot of the p85 immunoprecipitate was subjected to western blot analysis with the same antibody used for immunoprecipitation, to ensure that protein G pulls down the same amount of the enzyme in both samples (Fig. 1D). In order to investigate the precise cellular localization of PI 3-kinase, we performed western blot analysis



**Fig. 1.** Presence and activity of PI 3-kinase in human spermatozoa. Immunoprecipitation of (A) p85 regulatory subunit and (B) p110 catalytic subunit of PI 3-kinase from human sperm lysates and of MCF-7 or PC3 cells, respectively, as positive controls. Immunobeads obtained with anti-p85 PI 3-kinase or anti-p110 PI 3-kinase antibodies (IP) were separated by 8% SDS-PAGE and the transferred proteins were revealed by western blotting with the same antibodies used for immunoprecipitation (IB). Molecular mass markers are indicated on the right (A) and on the left (B) of the blots. (C) PI 3-kinase activity in sperm samples treated or not with 10  $\mu$ M LY294002 was evaluated by *in vitro* assay after immunoprecipitation with anti-p85 antibody. The spots correspond to PI 3-kinase catalytic product [ $^{32}$ P]phosphatidylinositol phosphate (PIP). Representative of four similar experiments. (D) Aliquot (1:4) of the p85 immunoprecipitates were subjected to western blot analysis with the same antibody, to ensure that protein G pulls down the same amount of the enzyme in both samples. Total lysates from PC3 cells and spermatozoa were run as positive controls.



with the antibody against the p85 regulatory subunit on purified sperm tail and head fractions. As shown in Fig. 2A, the regulatory subunit is mainly localized in sperm tails, although a weak positivity is also found in the heads after long exposure of the film, as confirmed by immunofluorescence analysis using the same antibody on fixed and permeabilized



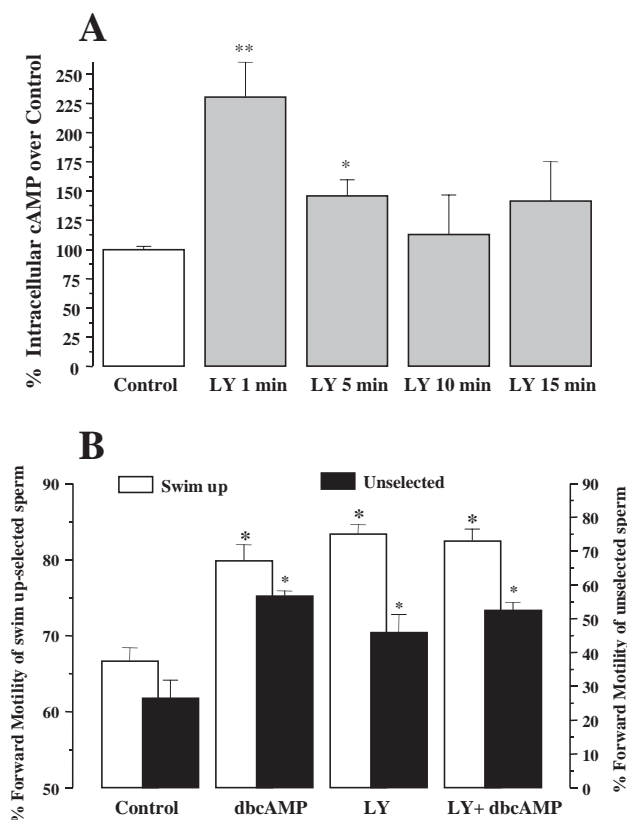
spermatozoa (Fig. 2B, b). No staining was present in sperm tails in the negative control (Fig. 2A, a).

In order to demonstrate that the stimulatory effect induced by LY294002 on sperm forward motility (Luconi et al., 2001) is caused by specific inhibition of PI 3-kinase, we compared the effects of LY294002 with those of the inactive analogue LY303511 (Vlahos et al., 1994). Direct addition of LY303511 to swim up-selected spermatozoa does not affect the percentage of forward sperm motility (Table 1), suggesting that the increase in motility induced by LY294002 is due to a specific inhibition of PI 3-kinase activity.

#### Involvement of cAMP and tyrosine phosphorylation of sperm proteins in mediating LY294002 stimulatory effect on motility

In order to elucidate the molecular mechanism by which inhibition of PI 3-kinase leads to stimulation of sperm motility, we investigated the two main intracellular signaling pathways involved in regulation of sperm motility, namely cAMP and

**Fig. 2.** Intracellular localization of PI 3-kinase in human spermatozoa. (A) Western blot analysis of tail and head protein extracts from swim-up-selected spermatozoa separated onto 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first blotted with anti-p85 PI 3-kinase regulatory subunit antibody (upper panel). Anti-ERK2 antibody (lower panel) was used to check for equal lane loading after stripping and re-probing of the same membrane. MCF7 cell lysate was used as positive control. Molecular mass markers are indicated on the right of the blot. Representative of two similar experiments. (B) Immunofluorescence analysis of fixed and permeabilized human spermatozoa probed with the antibody against the anti-p85 regulatory subunit of PI 3-kinase (b). Negative control avoiding the primary antibody is shown in a, whereas the respective fields observed in light transmission microscopy are reported in c and d. Arrows indicate sperm heads. Representative of four similar experiments.



**Fig. 3.** (A) Time-dependent effect of LY294002 on intracellular cAMP levels in swim-up-selected human spermatozoa. After different times of incubation with LY294002 (10  $\mu$ M), sperm samples were washed and extracted in ethanol. Intracellular cAMP concentrations were evaluated by a RIA kit as described in Materials and Methods. Data represent means $\pm$ s.e.m. from eight different experiments. \* $P$ <0.01, \*\* $P$ <0.001 versus control. Student's  $t$ -test for paired data. (B) Effects of dbcAMP and LY294002 on sperm motility. Swim-up-selected sperm samples were incubated for 1 hour in the presence of dbcAMP (1 mM) or LY294002 (10  $\mu$ M) alone or in combination and forward and rapid motility was evaluated by CASA. Data represent means $\pm$ s.e.m. from five different experiments. The percentage of sperm forward motility for swim-up-selected spermatozoa is indicated on the left ordinate, whereas the right ordinate indicates the same parameter for unselected spermatozoa. \* $P$ <0.001 versus respective controls, Student's  $t$ -test for paired data.

tyrosine phosphorylation of sperm proteins (Leclerc et al., 1996; Vijayaraghavan et al., 1997a; Carrera et al., 1996; Si and Okuno, 1999; Patil et al., 2002; Leclerc and Goupil, 2002). As shown in Fig. 3A, LY294002 stimulates a rapid and significant increase in intracellular cAMP levels in swim-up-selected spermatozoa after 1 and 5 minutes of incubation. The increase in cAMP is transient and declines to basal levels after 10 minutes of incubation, compared to control. Intracellular cAMP levels in basal condition in swim-up-selected spermatozoa were  $13.0\pm 4.4$  pmol/ $10^7$  spermatozoa ( $n=18$ ). Fig. 3B shows the effect of the cAMP analogue dibutyryl cAMP (dbcAMP) and of LY294002 on swim-up-selected and unselected spermatozoa. The two compounds stimulate a similar increase in sperm motility, without any synergic effect, suggesting that both compounds act on the same signaling pathway (Fig. 3B).

**Table 1.** Effect of addition of LY294002 or LY303511 to swim up selected spermatozoa on sperm forward motility

% Forward motility	Control	LY294002	LY303511
Swim up	61.8 $\pm$ 4.3	77.6 $\pm$ 5.9*	65.0 $\pm$ 5.1

Sperm forward motility (type a+b motility, according to WHO criteria, WHO, 1999) was evaluated by CASA in swim-up-selected spermatozoa incubated for 1 hour in the presence of LY294002 (10  $\mu$ M) or LY303511 (10  $\mu$ M). Data represent means $\pm$ s.e.m. of sperm forward motility in 10 different samples.

\* $P$ <0.001 versus control. Student's  $t$ -test for paired data.

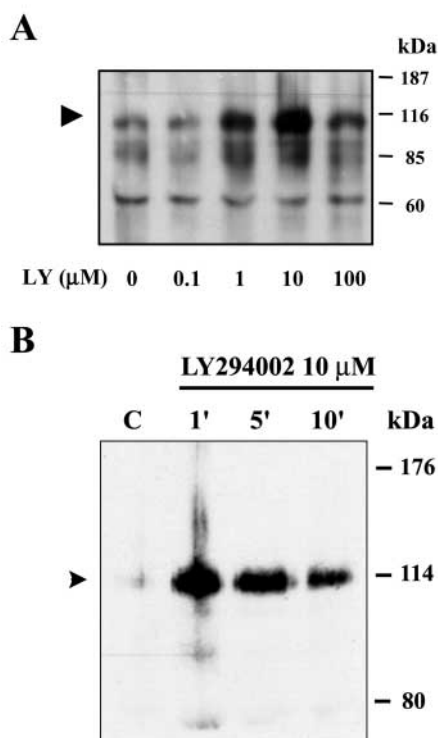
**Table 2.** Time-dependent effect of LY294002 on sperm motility in swim-up-selected human spermatozoa

% Motility		3 minutes	10 minutes	60 minutes
Forward	Control	63.1 $\pm$ 5.6	68.1 $\pm$ 3.0	62.1 $\pm$ 4.2
	LY294002	76.7 $\pm$ 5.8*	79.4 $\pm$ 2.4*	78.0 $\pm$ 3.3*
Rapid	Control	45.4 $\pm$ 8.1	48.6 $\pm$ 7.0	43.8 $\pm$ 7.8
	LY294002	55.7 $\pm$ 8.5*	63.9 $\pm$ 5.9*	57.07 $\pm$ 6.4*

Percentage sperm forward and rapid motility (type a+b and a motility respectively, according to WHO criteria; WHO, 1999) was evaluated by CASA in swim-up-selected spermatozoa incubated for different times in the presence of LY294002 (10  $\mu$ M). Data represent means $\pm$ s.e.m. of 13 different experiments.

\* $P$ <0.001 vs respective control, Student's  $t$ -test for paired data.

Since an increase in cAMP levels has been described to be associated with an increase in tyrosine phosphorylation of sperm proteins (Leclerc et al., 1996; Vijayaraghavan et al., 1985; Vijayaraghavan et al., 1997a; Leclerc and Goupil, 2002), we next evaluated the effect of LY294002 on the latter process. Western blot analysis of sperm lysates using PY20-HRP antibody shows that stimulation of swim-up-selected spermatozoa with LY294002 for 1 hour dose-dependently stimulates tyrosine phosphorylation of a sperm protein of about 110 kDa (Fig. 4A). An increase in tyrosine phosphorylation of a p110 kDa sperm protein band is already present after 1-minute stimulation with LY294002 (Fig. 4B), but, while the increase in cAMP levels is transient (Fig. 3A), the one in tyrosine phosphorylation appears to be long lasting. In view of such rapid effects of LY294002 on intracellular signal transduction events, we evaluated whether the stimulatory effect on sperm motility was also present at early times. As shown in Table 2, the effect of LY294002 on both forward and rapid motility is already present after a 3-minute stimulation and lasts for a long time. The involvement of tyrosine phosphorylation of sperm proteins in mediating LY294002 effects on sperm motility is also confirmed by the results obtained with erbstatin, a potent tyrosine kinase inhibitor. Indeed, as previously demonstrated (Luconi et al., 1995; Luconi et al., 1996), incubation of swim-up-selected spermatozoa with erbstatin reduces sperm forward motility and tyrosine phosphorylation of sperm proteins. In the presence of erbstatin (1 hour) the positive effects of LY294002 (10  $\mu$ M) on sperm motility are totally suppressed (Fig. 5A). This effect is only partially due to a toxic effect of erbstatin, since after 2-hour incubation only a slight decrease in sperm viability is observed (percentage of viability: control,  $89.1\pm 1.4$ ; erbstatin 25  $\mu$ g/ml,  $70.6\pm 6.0$ ; erbstatin 12.5  $\mu$ g/ml,  $70.3\pm 9.0$ ,  $n=6$ ; respectively 21.4% and 20.0% for viability decrease versus

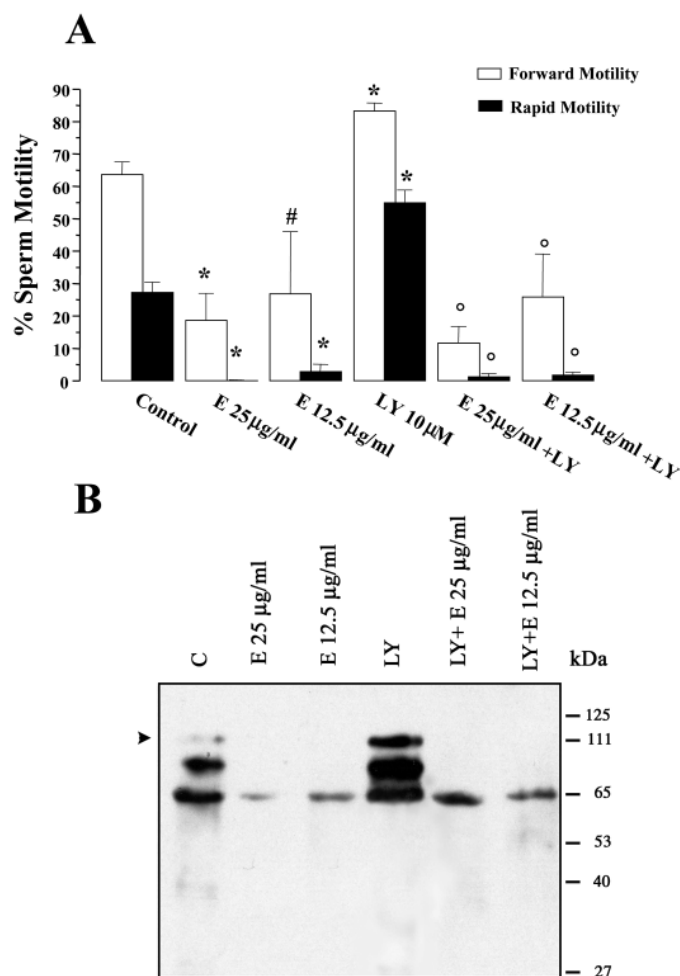


**Fig. 4.** Dose- and time-dependent effects of PI 3-kinase inhibition on tyrosine phosphorylation of sperm p110 kDa protein. Western blot analysis of sperm lysates from swim-up-selected spermatozoa stimulated for 1 hour with increasing concentrations of LY294002 (LY, A), or treated with 10  $\mu$ M LY294002 for the different times indicated (B) were separated by 8% SDS-PAGE. Equal amounts (30  $\mu$ g) of sperm proteins were subjected to 8% SDS-PAGE. Tyrosine phosphorylated proteins were revealed with PY20-HRP antibody. C, control at 10 minutes. Molecular mass markers are indicated on the right of the blots. Arrowheads indicate the modulated band at about 110 kDa. Representative of three similar experiments.

74.3% and 58.9% for motility decrease). Western blot analysis of protein extracts using PY20 antibody as probe shows that erbstatin also inhibits LY294002-stimulated increase in tyrosine phosphorylation of the p110 kDa protein band (Fig. 5B).

#### LY294002 induces an increase in tyrosine phosphorylation of AKAP 3 in sperm tails

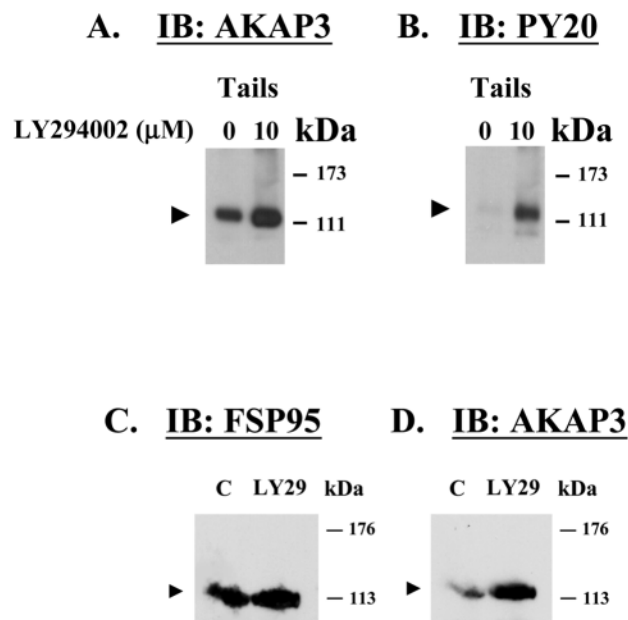
To characterize the nature of the 110 kDa band which is tyrosine phosphorylated following LY294002 stimulation (Fig. 4A,B; Fig. 5B), we performed western blot analysis of sperm lysates using a polyclonal antibody against AKAP3 [anti-AKAP3 antibody (Vijayaraghavan et al., 1999)]. This protein, of about 110 kDa molecular mass, belonging to the wide family of protein kinase A anchoring proteins (AKAPs), has been recently described in human spermatozoa as a scaffolding protein associated with the fibrous sheath of the flagellum, and is involved in regulation of sperm motility (Vijayaraghavan et al., 1999; Moss and Gerton, 2001). Since AKAP3 localizes predominantly in sperm tails, we performed western blot analysis of purified tail fractions from human spermatozoa treated or not with LY294002. Fig. 6A confirms that AKAP3



**Fig. 5.** Tyrosine phosphorylation of sperm p110 kDa protein mediates LY294002 stimulatory effects on motility. (A) Effects of 1 hour treatment with LY294002 (LY, 10  $\mu$ M) in the presence or absence of the tyrosine kinase inhibitor erbstatin (E, 12.5 or 25  $\mu$ g/ml) on both forward and rapid motility of swim-up-selected spermatozoa. Sperm forward motility and rapid motility was evaluated by CASA. Data represent means  $\pm$  s.e.m. of five different experiments. # $P$ <0.01 and \* $P$ <0.001 versus respective control, ° $P$ <0.001 versus respective LY; Student's  $t$ -test for paired data. (B) Western blot analysis of sperm lysates from swim-up-selected spermatozoa stimulated for 1 hour with 10  $\mu$ M LY294002 and erbstatin (E, 12.5 or 25  $\mu$ g/ml) together or alone. Equal amount (30  $\mu$ g) of sperm proteins were subjected to 8% SDS-PAGE. Tyrosine phosphorylated proteins were revealed in chemiluminescence with PY20-HRP antibody. Molecular mass markers are indicated on the right of the blots. Arrowhead indicates the p110 kDa protein. Representative of five different experiments.

is present in human sperm tails, as previously described (Vijayaraghavan et al., 1999). The stripping and re-probing of the same membrane with PY20 antibody reveals an increase in tyrosine phosphorylation in response to LY294002 in a p110 kDa protein band co-migrating with AKAP3 (Fig. 6B) on the same gel. The apparent increase in AKAP3 immunoreactivity observed in the lysates of spermatozoa treated with LY294002 (Fig. 6A) is evident in all the experiments (not shown) performed using the antibody against the human AKAP3 (see also Fig. 6D and Fig. 11B), although normalization of the





**Fig. 6.** LY294002-induced tyrosine phosphorylation of AKAP3. (A,B) Western blot analysis of tail protein extracts from swim-up-selected spermatozoa treated or not with 10  $\mu$ M LY294002, separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first blotted with anti-AKAP3 antibody (A) and, after stripping, re-probed with anti-phosphotyrosine antibody, PY20-HRP (B). (C,D) Western blot analysis of total protein extracts from swim-up-selected spermatozoa treated or not with 10  $\mu$ M LY294002. Twenty  $\mu$ g of sperm lysates were run in duplicate onto 8% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was cut longitudinally in two parts that were probed with anti-FSP95 (C) and anti-AKAP3 (D) respectively. Molecular mass markers are indicated on the right of the blots. Representative of three similar experiments.

blots with an unrelated antibody directed against a constitutive protein such as extracellular signal-regulated kinase 2 (Luconi et al., 1998), ERK2, does not reveal any difference in the protein loading (Fig. 11D). Since spermatozoa are transcriptionally and transductionally inactive, we speculate that the observed increase in AKAP3 positivity in the presence of LY294002 may be due to a stronger reaction of the anti-AKAP3 antibody with the tyrosine phosphorylated form of the protein. This hypothesis is also supported by the apparent

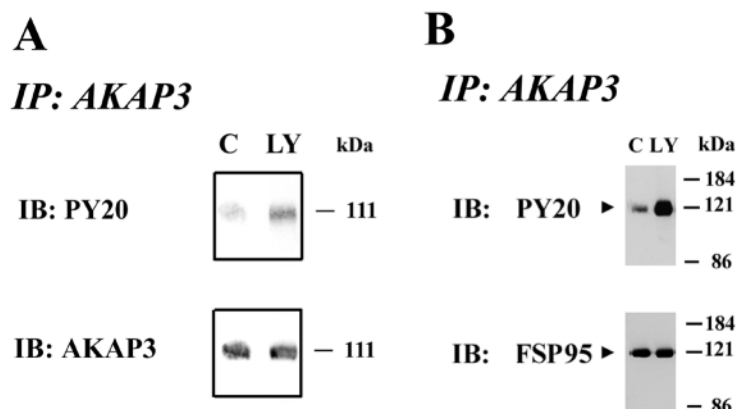
decrease in AKAP3 immunoreactivity in western blot analysis in the presence of erbstatin (Fig. 11B). On the other hand, a different antibody directed against AKAP3, anti FSP95 antibody (Mandal et al., 1999), does not reveal any increase in protein positivity in a 110 kDa band upon treatment with LY294002 (Fig. 6C, see also Fig. 11C), while such an increase was present when the same membranes were probed with the anti-AKAP3 antibody (Fig. 6D and Fig. 11B).

In order to further demonstrate that the 110 kDa protein undergoing tyrosine phosphorylation in response to LY294002 stimulation is AKAP3, we immunoprecipitated the protein with anti-AKAP3 antibody from spermatozoa treated or not with LY294002 (Fig. 7A,B). Western blot analysis using PY20 antibody as probe shows a marked increase in tyrosine phosphorylation of AKAP3 immunoprecipitated from LY294002 treated samples (Fig. 7A,B, upper panels). The stripping and re-probing of the same membrane with anti-AKAP3 (Fig. 7A, lower panel) and with anti-FSP95 (Fig. 7B, lower panel) antibodies shows that the anti-AKAP3 antibody pull down the same amount of proteins and that both anti-AKAP3 and anti-FSP95 antibodies do recognize the same protein (Vijayaraghavan et al., 1999; Mandal et al., 1999). The absence of any increase in AKAP3 positivity in LY294002-treated sample versus control using anti-AKAP3 antibody in western blot after immunoprecipitation (Fig. 7A), may be due to the AKAP3 extraction method used for immunoprecipitation compared to the simple lysis buffer used for western blot analysis (Fig. 6A and Fig. 11B).

#### The increase in tyrosine phosphorylation in AKAP results in tail recruitment of PKA through interaction with RII $\beta$

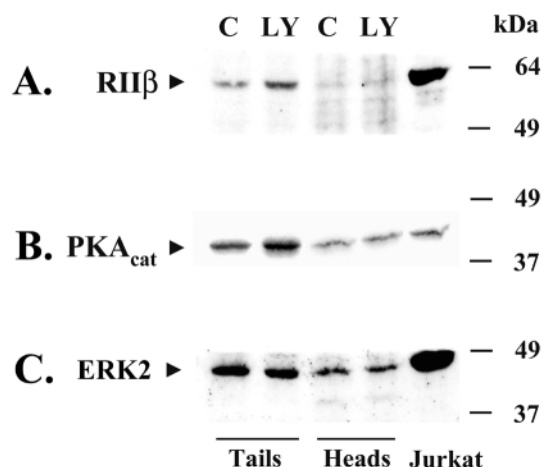
Western blot analysis of purified sperm tail and head fractions using specific antibodies against the regulatory RII $\beta$  (Fig. 8A) and the catalytic (Fig. 8B) subunits of PKA shows that both subunits are localized in both sperm tails and heads and that their positivity is increased in the former compartment following treatment of sperm with LY294002 (Fig. 8A,B).

To demonstrate that the observed increase of RII $\beta$  positivity in sperm tails following LY294002 stimulation (Fig. 8B) is due to an increased binding to AKAP3, we used Ht31, a pharmacological disruptor of this interaction (Vijayaraghavan et al., 1997b). Incubation of swim-up-selected spermatozoa with Ht31 results in a significant inhibition of both forward and rapid motility of sperm, which is totally prevented by LY294002 (Fig. 9A). The inactive inhibitor, P-Ht31, used as negative control, is ineffective (Fig. 9A). By interfering with PKA-AKAP interaction through RII $\beta$ , Ht31 induces



**Fig. 7.** Western blot analysis of immunoprecipitated AKAP3 from spermatozoa treated or not with 10  $\mu$ M LY294002. After 1-hour treatment, sperm samples were extracted in SDS buffer (see Materials and Methods). Cells lysates were immunoprecipitated (IP) using an anti-AKAP3 antibody, electrophoresed on 8% SDS, and immunoblotted with anti-phosphotyrosine antibody (IB: PY20-HRP, upper panels), stripped and re-probed with anti-AKAP3 antibody (IB: AKAP3, A, lower panel) or anti-FSP95 antibody (IB: FSP95, B, lower panel). Molecular mass markers are indicated on the right of the blots. Representative of three similar experiments.





the release of the PKA regulatory subunit from the insoluble fraction of sperm proteins to the soluble fraction, as demonstrated in bovine spermatozoa (Vijayaraghavan et al., 1997b). In western blot analysis of insoluble protein extracts from swim-up-selected human spermatozoa treated or not with 10  $\mu$ M LY294002, anti-RII $\beta$  antibody reveals an increase of RII $\beta$  following LY294002 stimulation both in the presence and absence of Ht31 (Fig. 9B, upper panel).

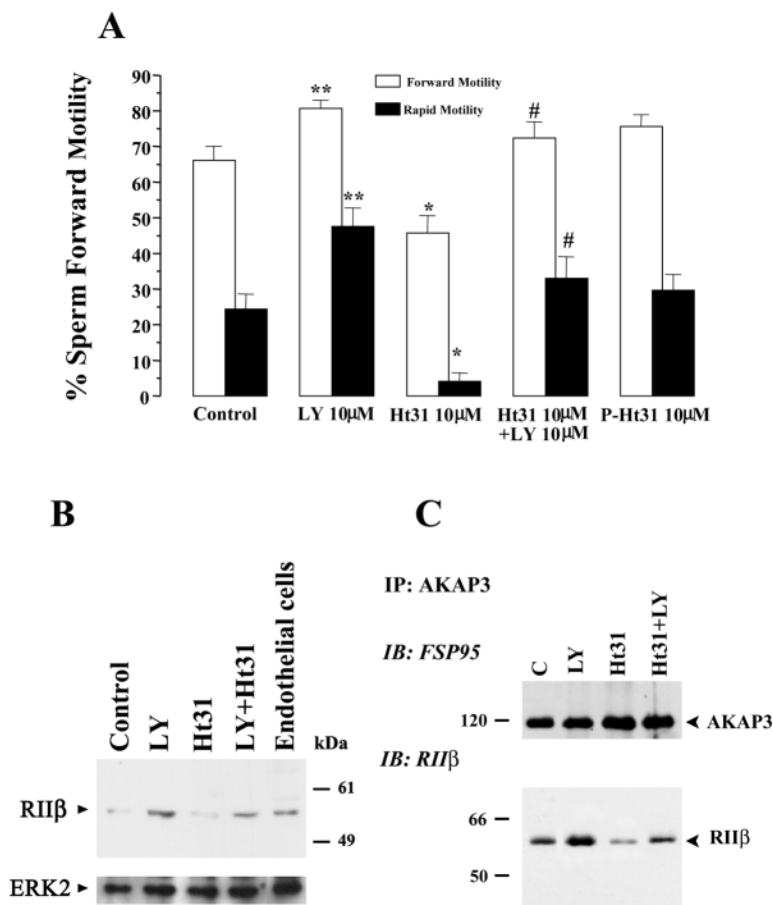
Co-immunoprecipitation experiments in which sperm protein extracts immunoprecipitated for AKAP3 were revealed in western blot analysis with anti-RII $\beta$  antibody to detect the

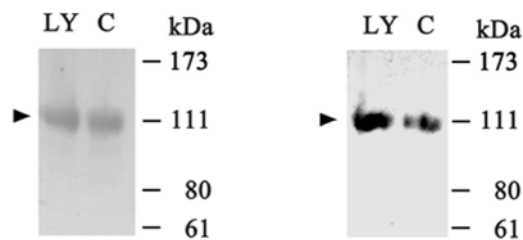
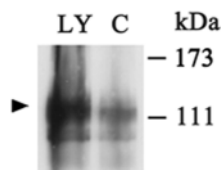
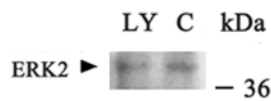
**Fig. 8.** Effect of LY294002 on localization of PKA regulatory and catalytic subunits in sperm tails and heads. Western blot analysis of tail and head protein extracts from swim-up-selected spermatozoa treated or not with 10  $\mu$ M LY294002, separated onto 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with antibodies against RII $\beta$  (A) and PKA catalytic (B) subunits. Anti-ERK2 antibody (C) was used to check for equal lane loading after stripping and re-probing of the same membrane. Jurkat cells have been used as positive controls for PKA subunits. Molecular mass markers are indicated on the right of the blots. Representative of three similar experiments.

amount of RII $\beta$  bound to AKAP3, show that LY294002 increases the interaction between AKAP3 and RII $\beta$ , as demonstrated by the increased amount of RII $\beta$  co-immunoprecipitated with AKAP3 in LY-stimulated samples (Fig. 9C). Moreover, the concomitant presence of LY294002 partially reverses Ht31 reduction in RII binding, demonstrating that LY294002 overcomes the inhibition exerted by Ht31 on AKAP3-RII $\beta$  binding.

To further demonstrate that the binding of RII $\beta$  to AKAP3 is increased by LY294002 in our cell system, we performed RII overlay assay on protein extracts from swim-up-selected spermatozoa treated or not with 10  $\mu$ M of the inhibitor. RII overlay assay demonstrates that following LY294002 stimulation there is an increase in the binding of RII $\beta$  to AKAP3 (average fold increase:  $2.3 \pm 0.3$  LY294002 vs basal,  $n=5$ ), but not of the RII $\alpha$  isoform (Fig. 10A,B). Stripping and reprobing the same membrane using anti-phosphotyrosine

**Fig. 9.** Effects of Ht31 on LY294002-stimulated increase in sperm motility, RII $\beta$  localization and interaction with AKAP3 in spermatozoa. (A) Swim-up-selected spermatozoa were incubated for 1 hour in the presence of LY294002 (10  $\mu$ M) with or without the active inhibitor Ht31 (10  $\mu$ M) or the inactive molecule P-Ht31 (10  $\mu$ M) and sperm forward and rapid motility was evaluated by CASA. Data represent means  $\pm$  s.e.m. of seven different experiments. \* $P < 0.01$  and \*\* $P < 0.001$  versus respective controls, # $P < 0.001$  versus Ht31, Student's *t*-test for paired data. (B) Western blot analysis of insoluble protein extracts from swim-up-selected spermatozoa treated or not with 10  $\mu$ M LY294002 in the presence or absence of 10  $\mu$ M Ht31. Insoluble protein fractions separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane were revealed first with anti-RII $\beta$  antibody (upper panel) and, after stripping, with anti-ERK2 antibody to check for equal lane loading (lower panel). Human endothelial cells were used as positive control. Molecular mass markers are indicated to the right of the blot. Representative of three similar experiments. (C) Effects of Ht31 and LY294002 on RII $\beta$ -AKAP3 interaction as revealed by co-immunoprecipitation of RII $\beta$  with AKAP3. Swim-up-selected spermatozoa treated with Ht31 or LY294002 alone or in combination were extracted in immunoprecipitation buffer containing 0.1% SDS and immunoprecipitated with anti-AKAP3 antibody (see Materials and Methods). After 8% SDS-PAGE of the immunobeads, proteins transferred to nitrocellulose membranes were revealed with anti-FSP95 (upper panel) or anti-RII $\beta$  (lower panel) antibodies. Molecular mass markers are indicated on the left of the blot. Representative of two similar experiments.



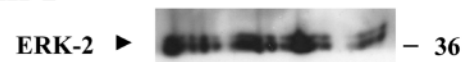
**A. Overlay Assay: RII $\alpha$**  **B. Overlay Assay: RII $\beta$** **C. IB: PY20****D. IB: ERK-2**

**Fig. 10.** RII overlay assay (A,B) and western blot analysis (C,D) of protein extracts from swim-up-selected spermatozoa treated or not with LY294002. After treatment with 10  $\mu$ M LY294002, sperm samples were lysed and proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. RII overlay assay: For RII overlay assay, membranes were first incubated with purified RII $\alpha$  (A) or RII $\beta$  (B) PKA regulatory subunits followed by probing with anti-RII $\alpha$  (A) or anti-RII $\beta$  (B) antibodies. After stripping, the same membrane was subsequently subjected to western blot analysis using PY20 antibody (C) and, finally, anti-ERK2 antibody (D) to check for equal protein loading (IB: western blot analysis). Arrowheads in A-C indicate the AKAP3, which is tyrosine phosphorylated following LY294002 incubation and which binds PKA RII regulatory subunits. Molecular mass markers are indicated on the right of the blots. Representative of three similar experiments.

PY20 (Fig. 10C) confirms the increase in tyrosine phosphorylation of AKAP3 following LY294002 treatment.

To gain insight into whether the increase in tyrosine phosphorylation of AKAP3 stimulated by LY294002 is involved in AKAP binding to RII $\beta$ , we incubated spermatozoa with 25  $\mu$ g/ml erbstatin for 1 hour and evaluated RII $\beta$  binding to AKAP3 by overlay assay. As shown in Fig. 11A, sperm treatment with erbstatin completely abolishes binding of RII $\beta$  to AKAP3 both in basal condition and in the presence of LY294002 (10  $\mu$ M), suggesting that tyrosine phosphorylation is necessary for AKAP3-RII $\beta$  interaction. After stripping, the same membranes were subsequently subjected to western blot analysis using anti-AKAP3 (Fig. 11B) and anti-FSP95 (Fig. 11C) to reveal the protein band interacting with RII $\beta$ .

Finally, the role of PKA activation in mediating LY294002 stimulatory effect on sperm motility was investigated by using the PKA inhibitor H89. Treatment of swim-up-selected spermatozoa with 50  $\mu$ M H89 reduces not only basal but also LY294002 and dbcAMP-stimulated motility to a similar extent (Fig. 12). However, the effect of the two compounds was still present in the presence of H89. This result suggests that, although PKA activation is necessary for both LY294002 and dbcAMP stimulation of sperm motility, the two compounds may act on multiple points on the cAMP/PKA pathway, as also demonstrated by their effect on tyrosine phosphorylation of

**A. RII  $\beta$  Overlay Assay****B. IB: AKAP3****C. IB: FSP95****D. IB: ERK-2**

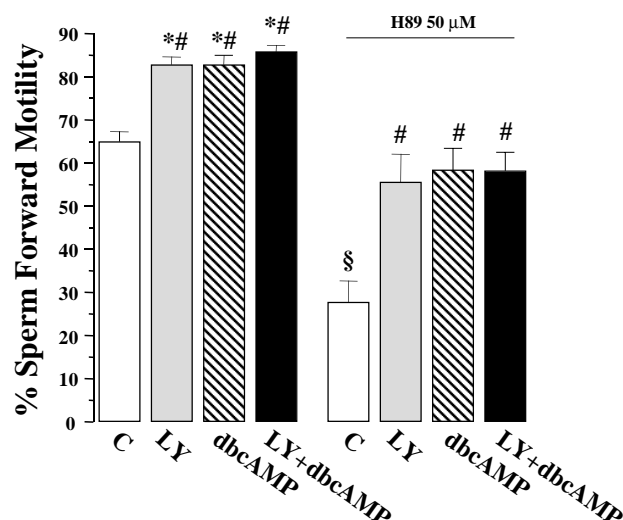
**Fig. 11.** Effect of inhibition of tyrosine phosphorylation of proteins by erbstatin on functional binding of RII $\beta$  to AKAP3 in human spermatozoa. Sperm proteins from spermatozoa treated with LY294002 (LY, 10  $\mu$ M) or erbstatin (E, 25  $\mu$ g/ml) alone or together for 1 hour, were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. (A) RII $\beta$  overlay assay: the membrane was incubated with purified RII $\beta$  subunit followed by probing with anti-RII $\beta$  antibody revealed by chemiluminescence. After stripping, the same membrane was subsequently probed with anti-AKAP3 (B, IB: AKAP3) and anti-FSP95 (C, IB: FSP95) antibodies. Arrowheads in A-C indicate the AKAP3, which is tyrosine phosphorylated following LY294002 incubation and which binds RII $\beta$  regulatory subunits. Anti-ERK-2 antibody was used to check for equal protein loading (D, IB:ERK-2). Molecular mass markers are indicated on the right of the blots. Representative of three similar experiments.

sperm proteins (these results) (Leclerc et al., 1996; Bajpai and Doncel, 2003).

**Discussion**

We have demonstrated that: (i) PI 3-kinase is present and active in human spermatozoa; (ii) pharmacological inhibition of PI 3-kinase by LY293002, previously shown to increase sperm motility (Luconi et al., 2001), induces a significant increase in intracellular cAMP levels and tyrosine phosphorylation of the PKA anchoring protein AKAP3; (iii) the increase in tyrosine phosphorylation of AKAP3 is necessary for PKA binding through recruitment of its regulatory subunit RII $\beta$  in sperm tails.

Although the presence of PI 3-kinase in human spermatozoa has been previously suggested by indirect studies employing pharmacological inhibitors such as wortmannin and LY294002 (Luconi et al., 2001; Fisher et al., 1998), direct evidence for its presence and activity has not been available. By immunoprecipitation, western analysis and immunofluorescence microscopy, we show here that both catalytic and regulatory subunits of the enzyme are present in human spermatozoa and that p85 mainly localizes in sperm



**Fig. 12.** Effect of PKA inhibition by H89 on sperm forward motility of swim-up-selected spermatozoa stimulated with LY294002 and dbcAMP. Swim-up-selected sperm samples were incubated for 1 hour in the presence of dbcAMP (1 mM), LY294002 (10  $\mu$ M) and H89 (50  $\mu$ M) alone or in combination, and forward and rapid motility were evaluated by CASA. Data represent means $\pm$ s.e.m. of five different experiments. \* $P$ <0.01 versus respective stimulus with H89 and # $P$ <0.01 versus respective controls (C) with or without H89; § $P$ <0.01 versus C without H89; Student's  $t$ -test for paired data.

tails. Immunokinase assay also shows that PI 3-kinase is active in human spermatozoa and is inhibited by LY294002. In addition, results of functional experiments demonstrating that LY294002, but not its inactive analogue LY303511, is able to increase sperm forward motility, provide evidence for the involvement of PI 3-kinase in regulation of this sperm function.

Increases in cAMP levels and protein phosphorylation are the main intracellular events involved in regulation of sperm motility (Leclerc et al., 1996; Vijayaraghavan et al., 1985; Vijayaraghavan et al., 1997a; Carrera et al., 1996; Si and Okuno, 1999; Patil et al., 2002; Leclerc and Goupil, 2002; Ficarro et al., 2003). We show here that inhibition of PI 3-kinase by LY294002 induces a rapid increase in both intracellular cAMP levels and tyrosine phosphorylation of AKAP3, a protein kinase A-anchoring protein that is mostly found in sperm tails. Both processes are associated with a rapid and sustained increase in sperm forward motility. The non-synergistic stimulatory effect on sperm motility exerted by LY294002 and dbcAMP strongly suggests that both compounds converge on the same signaling pathway of PKA activation. In agreement with our findings, inhibition of PI 3-kinase by LY294002 has been recently demonstrated to increase cyclic nucleotide-dependent excitation signaling in olfactory receptor neurons (Spehr et al., 2002).

Tyrosine phosphorylation of AKAP3 stimulated by LY294002 seems to be mediated by activation of sperm tyrosine kinases, since it is completely blocked by the tyrosine kinase inhibitor erbstatin, which also blocks the effects of LY294002 on sperm motility. LY294002 stimulation of tyrosine phosphorylation of AKAP3 is associated with an increased binding between AKAP3 and PKA as well as with a recruitment of both PKA regulatory and catalytic subunits to

the sperm tail compartment (Fig. 8B). Since both RII $\beta$  binding to AKAP3 and sperm forward motility are inhibited by treatment with the tyrosine kinase inhibitor erbstatin, tyrosine phosphorylation of AKAP3 seems to be necessary for its interaction with PKA. The involvement of increased PKA-AKAP3 interaction in the stimulatory effect of LY294002 on sperm motility is further substantiated by the ability of this compound to reverse the detrimental effect of Ht31 on motility (Fig. 9A). This compound, which has a hydrophobic sequence homologue to AKAP, has been widely demonstrated to disrupt RII binding to the anchoring protein, both in vitro, by overlay assay (Vijayaraghavan et al., 1997b; Carr et al., 1991; Carr et al., 1992) and NMR analysis (Newlon et al., 1999), and, in vivo, by fluorescence resonance energy transfer techniques (Ruehr et al., 1999). We show here that LY294002 is able to stimulate both RII $\beta$  recruitment to the sperm insoluble compartment (Fig. 9B) and RII $\beta$  binding to AKAP3 also in the presence of Ht31 (Fig. 9C), resulting in maintenance of sperm motility (Fig. 9A).

An increase in tyrosine phosphorylation during sperm capacitation has been previously described not only for AKAP3 (Vijayaraghavan et al., 1999; Mandal et al., 1999; Naaby-Hansen et al., 2002) but also for other members of the AKAP family, such as AKAP4 (Carrera et al., 1996), which localizes specifically to the fibrous sheath of the principal piece of sperm tail and shows high identity with AKAP3. Our study shows that tyrosine phosphorylation of AKAP3 determines an increased binding of RII $\beta$ , resulting in tail recruitment of PKA and increase of sperm motility, providing evidence for a functional role of tyrosine phosphorylation of AKAP3. PKA binding to tyrosine-phosphorylated AKAP3 is specifically mediated by RII $\beta$  and not by the  $\alpha$  isoform, suggesting that PKA activation is regulated not only by specific compartmentalization of the enzyme, but also by binding of different regulatory isoforms to the scaffolding proteins. Although the possible dual specificity nature of AKAP3 for both regulatory subunits type I and II has not yet been investigated, many AKAPs have been shown to possess a higher affinity for RII regulatory subunit than for RI (Burton et al., 1997). Thus, AKAP binds PKA-specific regulatory subunits to drive the holoenzyme to a defined compartment, allowing the catalytic subunit to interact with its substrates. The rapid and transient increase in intracellular cAMP levels induced by LY294002 in sperm suggests that PI 3-kinase inhibition not only results in the recruitment of PKA to sperm tails through tyrosine phosphorylation of AKAP3, but may also contribute to locally activate PKA. In fact, inhibition of PKA activity by H89, interferes with LY294002 stimulation of sperm motility. Whether AKAP-PKA interaction is also important for sperm PKA activation remains to be investigated. However, a recent report by Furusawa (Furusawa et al., 2002) demonstrated that disruption of AKAP-PKA interaction in HeLa cells results in suppression of PKA activation.

Despite several attempts to elucidate the role of different components of the PKA holoenzyme in regulation of sperm motility, it is not yet clear which regulatory subunit is important for sperm motility. In human sperm, disruption of RII $\beta$  interaction with AKAP3 dramatically inhibits motility (Vijayaraghavan et al., 1997b; Harrison et al., 2000), in agreement with our data. Conversely, in the mouse, deletion of RII $\alpha$  gene delocalizes the PKA catalytic subunit from the



insoluble to the cytosolic fraction, without, however, affecting sperm motility and fertilizing ability (Burton et al., 1999). However, the same group has recently reported that knockout mice for PKA $\alpha$  catalytic subunit show a concomitant decrease in RII $\alpha$  and  $\beta$  contents associated with a significant decrease in sperm forward motility, despite normal spermatogenesis (Skalhegg et al., 2002), thus suggesting a major role of PKA in regulation of sperm motility. These discrepancies may be due to species differences or may suggest a major role of RII $\beta$  rather than  $\alpha$  in modulating sperm motility. In this scenario, it is possible that disruption of AKAP3-PKA interaction, as a result of defects in the intracellular signaling (AKAP tyrosine phosphorylation) described in the present study, may account for alterations in sperm motility associated with male infertility. Tyrosine phosphorylation of AKAP3 might also regulate binding of other proteins probably involved in regulation of sperm motility, such as the recently described ropporin and AKAP-associated protein (Carr et al., 2001). The importance of AKAP scaffolding proteins in regulating sperm motility has been very recently brought to light by the finding that targeted disruption of the *Akap4* gene, whose product AKAP4 is close related to AKAP3, causes defects in sperm flagellum and motility in the mutant mice (Miki et al., 2002).

In conclusion, our data provide a novel molecular model according to which tyrosine phosphorylation of AKAP3, induced by inhibition of PI 3-kinase, stimulates a recruitment of PKA through its regulatory subunit RII $\beta$  to the sperm tail, resulting in an increase of sperm motility. Tyrosine phosphorylation of AKAPs may represent a more general mechanism for modulation of their affinity for different PKA regulatory subunits and other interacting proteins, thus leading to recruitment of these proteins to different cellular compartments.

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